(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 252 561**
**A2**

# (12) EUROPEAN PATENT APPLICATION

(21) Application number: 87201264.6

(51) Int. Cl.⁴: C12N 15/00 , C12P 21/00

(22) Date of filing: 03.07.87

The microorganism(s) has (have) been deposited with American type culture collection under number(s) ATCC 67024.

(30) Priority: 11.07.86 IT 2110686

(43) Date of publication of application:
13.01.88 Bulletin 88/02

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR LI LU NL SE

(71) Applicant: SCLAVO S.p.A.
Via Fiorentina 1
I-53100 Siena(IT)

(72) Inventor: Galeotti, Cesira
Strada Istieto 71
I-53100 Siena(IT)
Inventor: Palla, Emanuela
Via Adige 16
I-51016 Montecatini Terme Pistoia(IT)
Inventor: Raugei, Giovanni
Via Khioto 19
I-50126 Florence(IT)
Inventor: Bensi, Giuliano
Via Starnina 16
I-50143 Florence(IT)
Inventor: Melli, Maria Luisa
Via G. Duprè 65
I-53100 Siena(IT)

(74) Representative: Roggero, Sergio et al
Ing. Barzanò & Zanardo Milano S.p.A. Via Borgonuovo 10
I-20121 Milano(IT)

## (54) Expression and secretion vector in yeasts, useful for preparing heterologous proteins.

(57) Expression and secretion vector in yeasts, useful for preparing heterologous proteins, comprising a synthetic oligonucleotide, which directs the secretion of the heterologous protein, positioned between the inducible hybrid promoter GAL-CYC and a multiple-site polyinker followed by the signals of transcription termination recognized by the RNA-polymerase of the yeasts.

Hybrid plasmid obtained by cloning in one of the restriction sites of the polyinker of said vector, the DNA sequence which codes for a heterologous protein.

Process for the preparation of heterologous proteins, which comprises cultivating, in a suitable culture medium, a yeast transformed with said hybrid plasmid, and recovering from the culture medium the so-obtained proteins.

The so-obtained proteins comprise hormones, lymphoquins, viral antigens or immunogens, useful in the therapeutical and diagnostic field.

EP 0 252 561 A2

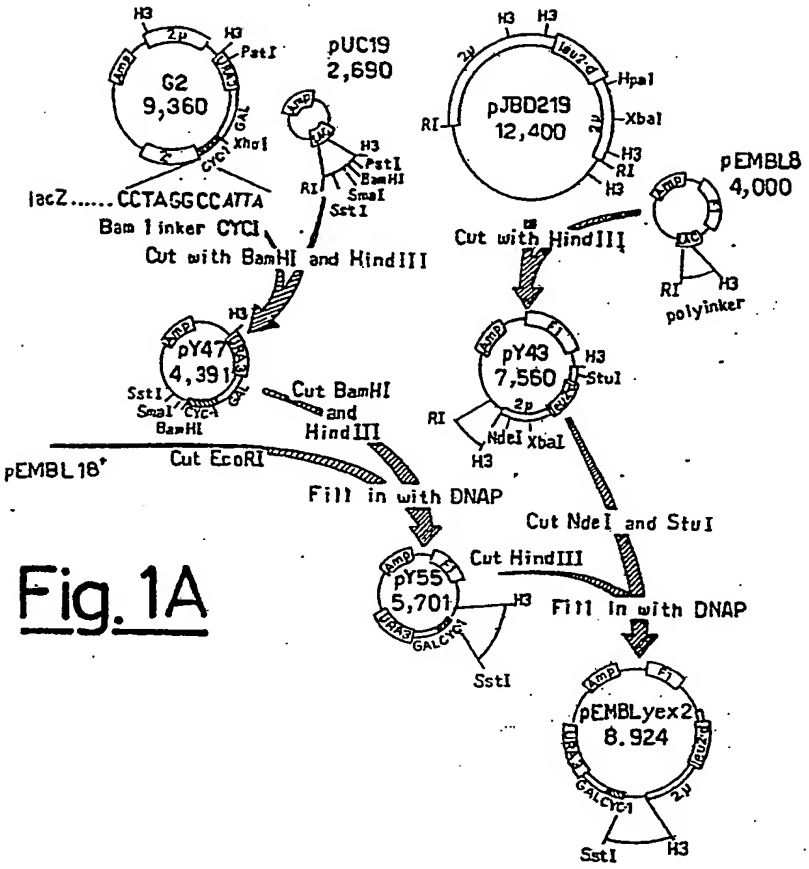

G2
9,360
pUC19
2,690
pJBD219
12,400
pEMBL8
4,000
polyinker
lacZ..... CCTAGGCCATTA
Bam linker CYC1
Cut with BamHI and HindIII
Cut with HindIII
pY47
4,391
pY43
7,560
Cut BamHI and HindIII
pEMBL18+
Cut EcoRI
Fill in with DNAP
Cut NdeI and StuI
Cut HindIII
pY55
5,701
Fill in with DNAP
pEMBLyex2n
8.924

Fig. 1A

# " EXPRESSION AND SECRETION VECTOR IN YEASTS, USEFUL FOR PREPARING HETEROLOGOUS PROTEINS".

The present invention relates to an expression and secretion cloning vector in yeasts, useful for preparing heterologous proteins.

The invention relates furthermore to a hybrid plasmid obtained by means of the union of said cloning vector with a DNA sequence which codes for a heterologous protein, and to a process for the preparation of said heterologous protein, which comprises cultivating, in a suitable culture medium, a yeast transformed by means of said hybrid plasmid.

The recent developments in the field of genetic engineering made it possible to prepare heterologous proteins by means of a process comprising the building, by means of recombinant DNA techniques, of a hybrid plasmid containing the gene coding for said protein, introducing said hybrid plasmid into host organisms, and finally cultivating the so-transformed organisms in a suitable culture medium for the purpose of obtaining the protein coded by the heterologus gene.

In general, the heterologous proteins have been prepared heretofore by using, as the host organisms, the bacteria, in that their regulation systems were known.

However, the use of bacteria in said processes causes various problems to occur, such as, e.g., those deriving from the production of enterotoxins, and from the limited availability of strains for use in industrial-type processes.

From the above, the need derives for having available host organisms which do not show the problems of the prior art, such as the yeasts, which, by being eucaryote organisms, show a greater affinity for mammals, and, differently from bacteria, do not produce enterotoxins, and have been long used in fermentation processes of industrial type.

However, for it to be possible a yeast to be used as the host cell, having available cloning vectors is necessary, which are stably kept inside the cells, and contain such regulation signals as to make it possible the cloned heterologous gene to be sufficiently expressed, and the genic product to be secreted in the culture medium.

From the technical and patent literature, processes are known for the building of expression and secretion vectors in the yeasts, which use regulatory sequences obtained from genes isolated from yeasts. In particular, vectors were built for the secretion of homologous and heterologous proteins, by using leader peptides involved in the secretion of the $\alpha$ factor and of the killer toxin of Saccharomyces cerevisiae (S. cerevisiae). However, said vectors, used in the preparation of human interferon (Singh et al., Nucl. Acids Res., 12, 8927-8938, 1984) and of murine interleukin IL-2 (Miyajima et al., Gene, 37, 155-161, 1985), give rise to a particular secretion of synthesized protein.

In fact, both the $\alpha$ factor and the killer toxin of S. cerevisiae have a leader peptide completely different from those typical for multicellular organisms.

Recent investigations carried out on Kluyveromyces lactis (K. lactis) yeast demonstrated that the killer strains belonging to this species secrete a toxin which is structurally and functionally different from the killer toxin of S. cerevisiae (Sugisaki, Y. et al., 1983, Nature 304, 464-466).

Said toxin results in fact to be constituted by two sub-units, and is secreted in the culture medium as a glycoprotein. At a later time, Stark et al. (Nucl. Acids Res., 12, 6011-6030, 1984), on the basis of the study carried out on the nucleotidic sequence of the gene coding for the toxin of K.lactis, hypothesized that its secretion could be directed by a 16-aminoacid long leader peptide, whose structure was similar to that of the leader peptides of procaryotes and eucaryotes.

Even if the presence of the regulation systems can be supposed, it is however not possible to predict whether such systems, used in the building of cloning vectors, are endowed with the capability of performing their functions.

A cloning vector was found now, which makes it possible an efficient expression and secretion of heterologous proteins in yeasts.

A purpose of the present invention is therefore an expression and secretion cloning vector in yeasts, useful for the preparation of heterologous proteins, wherein the secretion of the heterologous proteins synthesized is directed by a synthetic oligonucleotide, positioned between an inducible hybrid promoter, and a multiple-site polyinker, followed by the signals of transcription termination recognized by the RNA-polymerase of yeasts.

Another purpose of the present invention is a hybrid plasmid obtained by means of the union of said cloning vector and a DNA sequence coding for a heterologous protein, wherein said sequence is inserted in one of the restriction sites of the polyinker placed downstream the synthetic oligonucleotide.

A further purpose of the present invention is a process for the preparation of heterologous proteins, which comprises cultivating, in a suitable culture medium, a yeast transformed with said hybrid plasmid.

A still further purpose of the present invention is the use of the so-obtained heterologous proteins in the diagnostic and therapeutical fields.

Other purposes of the invention shall be clear to those skilled in the art from the following disclosure.

In accordance with the above, according to the present invention a secretion vector was built by inserting between the restriction sites SstI and KpnI of the expression vector pEMBLyex2 placed downstream an inducible hybrid promoter, the synthetic oligonucleotide which codes for the hypothetical leader peptide of the killer toxin of K. lactis.

In particular, said oligonucleotide comprises the coding section for 16 aminoacids of the amino-end of the killer toxin from the initiator methionine to the presumed site of the cleavage signal of endopeptidase Val-Gln-Gly (D. Perlman et al., J. Mol. Biol. 167, 391-409, 1983).

Said synthetic oligonucleotide is positioned between the inducible promoter (GAL-CYC) and the multiple-site polyinker of p-EMBL18 vector (L. Dente et al., DNA Cloning, Vol. I, IRL Press, London, U.K., pages 101-107, 1985). followed by the signals of transcription termination (3' end of FLP of 2 μm plasmid) of pEMBLyex2.

In particular, in said polyinker six individual recognition sites for the restriction endonucleases are present (SmaI, XmaI, BamHI, SalI, PstI, HindIII), which allow the insertion, downstream the synthetic oligonucleotide, of DNA sequences coding for interesting proteins, such as lymphoquins, hormones, viral antigens and immunogens.

The secretion vector according to the present invention, indicated as YEsec1, contains furthermore the necessary sequences for the selection and replication in E. coli, as well as the necessary elements for the selection and episomal replication in S. cerevisiae.

According to the present invention, the expression vector pEMBLyex2, the building of which is reported in Figure 1A and in Example 1, contains two blocks obtained from yeasts and the necessary sequences for the selection and replication in E. coli. The first block, which determines the episomal replication and the number of plasmid copies, derives from pJBD219 plasmid (Beggs, J.D., 1978, Nature 275, 104-109) and comprises the NdeI-StuI fragment of 3,220 base-pairs containing leu2-d, 2 μm STB and ORI portions of pJBD219. This fragment comprises, furthermore, a small portion of the 3' end of FLP gene of 2 μm plasmid containing the signals of termination of transcription and polyadenylation.

The second block corresponds to HindIII-BamHI fragment of G2 plasmid (Guarente L., 1983, Methods Enzymol., 101, 181-191) and contains the URA 3 gene and the signals which induce the transcription in the polyinker during the growth of the strains transformed with said vector in a medium containing galactose as the only carbon source. The capability of YEpsec1 to direct the expression and secretion of a heterologous gene was verified by cloning, in BamHI site of the polyinker situated immediately downstream the synthetic oligonucleotide of YEpsec1, the cDNA coding for aminoacids 121-269 of human interleukin 1β.

The so-obtained hybrid plasmid, YEpsec1-hIL1β, was then used for transforming cells of Saccharomyces cerevisiae, preferably S. cerevisiae S150-2B.

The so-transformed cells, said ThI1, were then cultivated, according to general techniques known from the prior art, in a culture medium, containing, as its only carbon source, either galactose or ethanol.

The electrophoretic analysis of the supernatants and of the whole cellular extracts of ThI1 and Tsec1, i.e., cells of S. cerevisiae S150-2B, transformed with the vector YEpsec1 lacking the DNA of human interleukin 1β, show the presence of a protein of about 22,000 daltons in the only supernatant of ThI1 cells (line C, figure 2), grown on galactose.

Furthermore, the immunoblotting analysis of said protein in the supernatant and in the cellular extracts of ThI1 cells cultivated in the presence of galactose shows a complete secretion of this protein in the culture medium, By assuming that the secreted protein is recombinant IL-1β, its molecular weight should be of about 17,000 daltons.

In as much as IL-1β contains in its sequence a potential site of glycosylation (Asn-Cys-Thr) at the amino-end, the discrepancy in molecular weight could be due to the presence of oligosaccharides bound to the amino group. Hence, according to the present invention, the secreted protein was digested with endoglycosidase H and the digestion product was than analysed again by gel-electrophoresis.

The results shown in Figure 2 show in fact that, after treatment with endoglycosidase H, the band corresponding to 22,000 daltons disappears and, as expected, a new band of 17,000 daltons appears.

This result was furthermore confirmed by means of the analysis of the supernatants of ThI1 cells grown in a medium containing galactose and tunicamycin, a glycolysation inhibitor.

According to the present invention, the sequence of the nine aminoacidic radicals from the amino end of the secreted protein was determined.

Said sequence (Ser-Leu-X-X-Thr-Leu-Arg-Asp-Ser) results to be in agreement with that specified from the 5' end of cDNA of IL-1$\beta$ (Auron et al., 1984, Proc. Natl. Acad. Sci. USA, 81, 7907-7911) cloned in YEpsec1.

Furthermore, it does not show the presence of anyone of the three aminoacids coded by the sequence of the polyinker positioned between the synthetic oligonucleotide and the DNA of IL-1$\beta$.

That indicates that the cleavage of the leader peptide takes place between the last one of the 3 aminoacids (Figure 1B, bottom, black arrow) specified by the sequence of the polyinker and the first aminoacid of IL-1$\beta$ and that the leader peptide results hence to be constituted by 19 aminoacids, i.e., the 16 aminoacids of the synthetic oligonucleotide of the hypothetical leader peptide of the killer toxin and the three aminoacids of the polyinker of pEMB18 (Thr, Arg, Gly).

In accordance with the present invention, the biological activity of the secreted recombinant protein was determined by means of the test of stimulation of proliferation of mouse thymocytes.

The results obtained, reported in Figure 4, show that said protein of 22,000 daltons is endowed with the same biological activity as of natural IL-1$\beta$. Cells of E. coli containing the hybrid plasmid YEpsec1-hl1 were filed on March 6th, 1986, with the American Type Center Culture as ATCC 67024.

## Brief Description of the Figures

### Figure 1A:

The building is schematically reported of the expression pEMBLyex2 plasmid vector and of the secretion YEsec1 vector.

The pY47 plasmid is built by linking the BamHI-HindIII fragment of 1,700 base-pairs (bp) of G2 plasmid to the BamHI-HindIII fragment of 2,690 bp of plasmid pUC19 (pY42) and removing from pY42 the PstI restriction site in URA3 gene by means of digestion with PstI and treatment with DNA-polymerase I Klenow fragment. The BamHI-HindIII fragment of 1,700 bp of pY47 is isolated and bound to plasmid pEMBL18, previously cut with EcoRI (pY55).

The pY43 plasmid is built by inserting in pEMBL8 the HindIII fragment of 3,560 bp of plasmid pJDB219 containing leu 2-d, STB of 2 µm plasmid and the sequence of the 3' end of FLP of 2 µm plasmid.

The NdeI-StuI fragment of 3,220 bp of pY43 is then linked to HindIII fragment of pY55 to yield the hybrid plasmid pEMBLyex2.

### Figure 1B

In this Figure, the map is reported of the secretion vector YEpsec1 obtained by inserting between the SstI and KpnI restriction sites of pEMBLyex2 the synthetic oligonucleotide.

The sequence reported in the lower part of Figure 1B corresponds (boldface characters) to the sequence of the hypothetical leader peptide of the gene of the Killer toxin of K.lactis from the initial methionine up to the presumed site of cleavage of endopeptidase (Val-Gln-Gly) (empty arrow), to the junction of this latter (lower-case letters) with the cDNA of interleukin-1$\beta$) (IL-1$\beta$) cloned in said vector (undelined).

The black arrow indicates the experimentally found cleavage site.

### Figure 2

Figure 2 reports the electrophoretic analysis on gel of SDS-PAGE polyacrylamide stained with Coomassie of the whole cellular extracts (a and b) and of the supernatants of the cultures (c and d) of Thl1 grown in a complete medium supplemented with 2% of galactose (a and c) or 2% of ethanol (b and d) and of the whole cellular extracts (e and f) and supernatatants of cultures of Tsec1 cells (g and h) grown in a complete medium supplemented with 2% of galactose (e and g) or with 2% of ethanol (f and g).

The 67.7-kd band present in all of the supernatants is the bovine seralbumin (BSA) used as the support for protein precipitation.

### Figure 3

Figure 3 reports the electrophoretic analysis on SDS-PAGE of the 22,000-dalton protein, before (a) and after (b) the treatment with endoglycosydase H.

### Figure 4

The chart shows the stimulation of the proliferation of mouse thymocytes by means of the supernatant fraction obtained from a culture of Thl1 grown in a medium with galactose.

Abscissae: Volume (ml) of elution from the Sephacryl-S200 column.

Ordinates: Incorporation of [$^3$H]-thymidine by the mouse thymocytes.

The incorporation stimulates by the only phytohemoagglutinine (PHA) is shown in the dotted area. The standard molecular weights are indicated by the arrows. Each point is the average of three determinations with SEM <10%.

Example 1

Building of the Expression and Secretion YEpsec1 Vector (Figures 1a and 1b of the Drawing Tables)

2 µg of G2 plasmid and 2 µg of pUC19 plasmid are respectively cut with 2 units (U) of restriction enzyme BamHI and HindIII (Biolabs) in 20 µl of phosphate buffer (pH 7.4), under the operating conditions as suggested by the supplier. The digestion reaction is then stopped at 65°C for 10 minutes, and the resulting digestion mixtures are charged on low-liquifying-point 0.6% agarose gel (BRL) and run at 110 V for 3 hours.

The bands respectively corresponding to the BamHI-HindIII fragment of 1,700 base-pairs (bp) of G2 and BamHI-HindII fragment of 2,690 bp of pUC19 are cut and linked in the presence of T4 DNA ligase, without isolating them from agarose, as described by Crouse et al., in Methods in Enzymology, Vol. 101, pages 78-89, 1983).

10 µl of the so-obtained ligase mixture are used for transforming cells of E.coli JM 101 (BRL), rendered competent according to the method as described by D.A. Morrison in Enzymol., Vol. 68, 326 (1979).

The transformants are selected on slabs of LB agar (DIFCO), to which 50 µg/ml of Ampicillin were added.

From one of the so-obtained positive clones, the pY42 plasmid, of 4,391 bp, is extracted. Said plasmid is cut with 2 U of PstI (BRL) and is treated with polymerase (Klenow-Boehringer) for 5 minutes at 37°C, for the purpose of eliminating the PstI restriction site in URA 3 gene.

The mixture is then treated with T4 DNA ligase in 1mM ATP, 10 mM dithiothreitol, 20 mM tris-HCl, 10 mM $MgCl_2$ buffer, at 15°C, for 16 hours and to an end concentration of 150 µg/ml of DNA.

The whole ligase mixture is then used for transforming competent cells of E-coli JM 101, as reported above.

From one of the positive clones, pY47 plasmid is isolated. This plasmid is cut with BamHI and HindIII, as previously reported, and the BamI-HindIII fragment of 1,700 bp of pY47 is linked to the pEMBL18 plasmid previously cut with EcoRI and treated with polymerase. The ligase mixture is then used for transforming competent cells of E. coli JM 101. From one of the positive clones, the pY55 plasmid of 5,701 bp is isolated.

The analysis of hybridization on cellulose filter shows that said plasmid is constituted by pEMBL18 and by the BamHI-HindIII fragment of 1,700 bp. 2 µg of pJBD 219 (J.D.Beggs, Nature, 275, 104-109 (1978) and 2 µg of pEMBL8 (L. Dente et al., DNA Cloning, Vol. 1, IRL Press, London, U.K., pages 101-107 (1985) are respectively cut with 2 U of HindIII (Biolabs).

The digestion mixture is then charged on 0.6% agarose gel and run at 110 Volt for 3 hours. The band corresponding to the HindIII fragment of 3,560 bp of pJBD19 (Baggs J.D., Nature 275, 104-109, 1978) is cut and added to the digestion mixture of pEMBL8 in the presence of T4 DNA ligase.

10 µl of ligase mixture is then used for transforming competent cells of E. coli JM 101 and from one of the positive transformants the pY43 plasmid of 7,560 bp is isolated.

2 µg of pY43 is digested with 2 U of NdeI and 2 U of StuI (BRL).

5 µl of said digestion mixture is added to 5 µl of a digestion mixture obtained by cutting pY55 with HindIII, in the presence of T4 DNA ligase, by operating as above.

The positive clones are identified by hybridization using as the probe the NdeI-StuI fragment of labeled pY43. By operating in such way, the hybrid pEMBLyex2 plasmid of 8,924 bp is isolated, whch contains the gene coding for Ampicillin resistance, the origin of replication of F1 phage, the gene of uracyl 3 (URA 3) and the leucine 2-d gene of S. cerevisiae and the hybrid promoter derived from G2 follwed by the polyinker of pEMBL18.

15 µg of pEMBLyex2 is cut with 5 U of SstI and partially with 1 U of KpnI. the digestion mixture is then charged on 0.6% agarose gel and run at 110 V for 3 hours.

The band corresponding to the highest-molecular-weight fragment is separated and linked with the oligonucleotide:

C ATC AAT ATA TTT TAC ATA TTT TTG TTT TTG CTG TCA TTC GTT CAA GGT AC TC GAG TAC TTA TAT AAA ATG TAT AAA AAC AAA AAC GAC AGT AAG CAA GTT C

synthesized by using an Applied Biosystems 380A synthesizer.

The ligase mixture is also used for transforming competent cells of E. coli HB101 (F⁻ hsdS20 rec A13 ara14 proA2 lacY1 galK2 rpsL20 xy15 met-1 supE44).

The transformants are then selected for Ampicillin-resistance as reported above.

From a positive clone, YEpsec1 plasmid is finally isolated (Figure 1B).

Example 2

Building of Hybrid YEpsec1-hI-1β plasmid

Cells of THP1 cellular line, grown in RPMI to a density of $10^6$ cells/ml, are stimulated for 4 hours with TPA (0.1 µg/ml). Total RNA is extracted from such cells according to the method as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring, Harbor, 1982, 194-195.

RNA is run on a column of oligo-dT and is fractionated on a saccharose gradient (5-20%) in 20 mM sodium acetate (pH 5.5). The fractions are hybridized with a synthetic probe of 55 bp corresponding to the last 18 codons of the sequence of the gene of interleukin-1β (IL-1β) (Auron et al., P.N.A.S. 81, 7908-7011, 1984).

The positive fractions are used for preparing the cDNA's according to as described by Gruber et al. in Gene 25, 263-269 (1983).

The above described probe is used for identifying the recombinant clones.

The plasmidic DNA of one of these clones is used for isolating the Sau3A partial fragment of 600 bp containing the cDNA of IL-1 (Auron, l. cit.). Such fragment is inserted in BamHI site of YEsec1 plasmid situated immediately downstream the synthetic oligonucleotide for obtaining the hybrid YEpsed1-hI-1β plasmid. This latter was used for transforming the cells of E. coli and these cells were filed on March 6th, 1986, with the American Type Center Culture with the access number ATCC 67024.

Example 3

Expression and Secretion of Interleukin-1β from cells of S. cerevisiae

Cells of S. cerevisiae S150-2B (leu 2-3 leu 2-112 ura 3-52 trp 1-289 his 3-1 cir$^+$) are respectively transformed with the hybrid YEpsec-hI1β plasmid (S. cerevisiae ThI1) and with the YEsec1 vector (S. cerevisiae Tsec1) according to the method by Rothstein R, (1985) in Glover, D.M. (Ed.), DNA Cloning, IRL Press, London, Vol. II, pages 45-46.

The so-transformed cells are cultivated inside Erlenmeyer flasks of 2 l of capacity, containing 1 l of a medium having the following composition:

-yeast extract (DIFCO) 1 %
-peptone (DIFCO) 1 %
-galactose (Merck) 2 %
-$KH_2PO_4$ 0.3%
- $H_2O$ 1 litre

(pH 5.5) at 200 rpm, and at 30°C.

After approximately 40 hours, the electrophoretic analysis of the supernatants and of the whole cellular extracts of Tsec1 and ThI1 is carried out, for the purpose of determining the presence of IL-1β.

The cellular extracts are prepared by mechanical cold-breakage of the cells by means of small glass balls, for 2 minutes inside a vortex, centrifuging of the cellular suspension at 10,000 rpm for 15 minutes, and then dilution of the so-clarified liquid in a buffer of 2% SDS, 10% glycerol, 5% mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8.

The supernatants are prepared on the contrary by centrifuging the cell culture at 4,000 rpm for 5 minutes and filtering the supernatant on sterile filters of 0.45 µm Millipore.

The filtrate is then stored at 0°C and is subsequently analysed for determining IL-1β.

To 1.8 ml of said filtrate, bovine seralbumin (BSA) by SIGMA, to an end concentration of 100 µ/ml, and 0.2 ml of trichloroacetic acid (TCA) are then added.

The mixture is then kept at -20°C for 30 minutes and is centrifuged at 10,000 rpm for 15 minutes. The precipitate is washed and re-suspended in 25 µl of buffer (2% sodium dodecyl sulphate, 10% glycerol, 5% mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8).

The obtained solution is maintained at boiling temperature for 3 minutes, and is then charged on 15% acrylamide gel, prepared as described by Laemmli, Nature (1970) 227, 680-684, and is run at 150 V for 5 hours.

For control purposes, the same analyses are carried out on the cellular extracts, and on the supernatant of ThI1 and Tsec1 cells cultivated in a complete medium, wherein galactose was replaced by ethanol, which, in the yeast, does not activate the expression of the gene which codes for human IL-1β.

The gel-electrophoretic analyses are carried out by using as the control standard for the molecular weights 1 µl of Low Molecular Weight by BIORAD (14,400-92,000 daltons).

The results reported in Figure 2 show the presence, in correspondence of 22,000 daltons, of a band in the filtrate from the culture of S. cerevisiae ThI1 only. By assuming that the protein secreted by ThI1 is IL-1β, its molecular weight should be of about 17,000, and not 22,000, daltons, and the discrepancy in the molecular weight should hence be due to the presence of oligosaccharides bound to the end amino group of the protein. The digestion of said protein with endoglycolase H enzyme shows in fact the disappearance of the band of 22,000 daltons, and the appearance of a new band of approximately 17,000 daltons (Figure 3).

This result is confirmed by means of the inhibition of the glycosylation in vivo by means of tunicamycin.

The supernatants of cultures of Thl1 grown in a medium containing galactose and tunicamycin at a concentration higher than 0.5 µg/ml produce in fact only the protein of 17,000 daltons.

For the purpose of verifying the identity of said protein, and the correct secretion processing, the first aminoacids are analysed according to the method by Edman, reported by Hewick et al in J. Biol. Chem., 256, 1990-1997 (1981). The results obtained confirm the expected sequence for IL-1β. None of the aminoacids coded by the polyinker is found at the amino end of the protein, thus suggesting that the cutting of the leader peptide takes place between the last one of the three aminoacids coded by the sequence of the polyinker and the first aminoacid of IL-1β (Figure 1B).

The biological activity of the so-secreted protein is tested on the supernatant obtained from the culture of Thl1 cells by means of the analysis of thymocyte proliferation, as described by Gerg et al. in Cell. Immunology, Vol. 64, 293-303 (1984).

In practice, the supernatant is concentrated by means of ultrafiltration and is charged on a Sephacryl-S 200 column (30×1 cm) equilibrated with 0.15 M NaCl at a flowrate of 1 ml/minute and fractions of 0.5 ml, with a molecular weight of from 20,000 to 22,000 are collected and analysed as follows.

$6\text{-}10^5$ thymocytes obtained from C3H/HeJ mice of 4-8 weeks are introduced in wells of microslabs, and are contacted with 0.2 ml of scalar solutions of the fractions in RPMI 1640 medium, in the presence of 5% phoetal seralbumin (FBS, Hy-Clone, Sterile Systems), 50 µg/ml of gentamycin sulphate (Sigma), 25 mM HEPES, 2mM L-glutamin and $1.25\times10^{-5}$ M of 2-mercaptoethanol.

The cultures are labeled with 1 µCi/well of 3HdThd thymidine and are maintained at 37°C for 16-18 hours. At the end of this time, the cells are recovered on fiberglass filters and analysed by spectrometry, for the purpose of determining the incorporated radioactivity. The proliferation is measured as counts per minute (cpm), and is expressed as a "proliferation index", i.e., the ratio of the PHA + fraction cpm to the control cpm (i.e., PHA without fraction). The results (Figure 4) indicate a high proliferation in correspondence of the fraction with a molecular weight of about 22,000 daltons.

## Claims

1. Expression and secretion cloning vector in yeasts, useful for preparing heterologous proteins, characterized in that a synthetic oligonucleotide, which directs the secretion of the heterologous protein, is positioned between the inducible hybrid promoter and a multiple-site polyinker followed by the signals of transcription termination recognized by the RNA polymerase of yeasts.

2. Vector according to claim 1; characterized in that the inducible hybrid promoter is GAL-CYC, the termination signals are those of the 3'-end region of FLP, the sequence of the synthetic oligonucleotide corresponds to the hypothetical leader sequence of the Killer toxin of K. lactis and the multiple-site polyinker is that of pEMBL18 vector.

3. Expression and secretion hybrid plasmid, obtained from the cloning vector according to claims 1 and 2 and from a DNA sequence coding for a heterologous protein, said sequence being cloned in one of the restriction sited of the polyinker downstream the synthetic oligonucleotide.

4. Hybrid plasmid according to claim 3, characterized in that said sequence is constituted by cDNA which codes for human interleukin-1β of BamHI of the polyinker immediately downstream the synthetic oligonucleotide.

5. Hybrid YEpsec1-hI-1β plasmid contained in the strain of E. coli, filed with the American Type Culture Center with the access number ATCC 67024.

6. Process for the preparation and the secretion of a heterologous protein which comprises the cultivation, in a suitable culture medium, of yeast cells containing the hybrid plasmid according to claims from 3 to 4.

7. Use of the heterologous proteins obtained by means of the process of claim 6 in the therapeutical and diagnostic field.

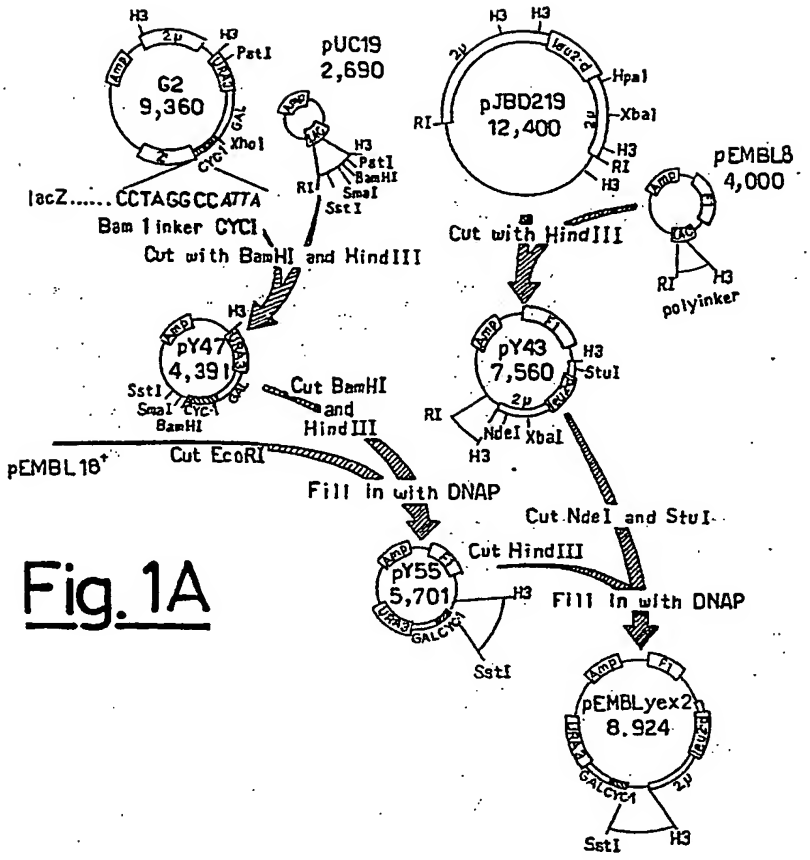
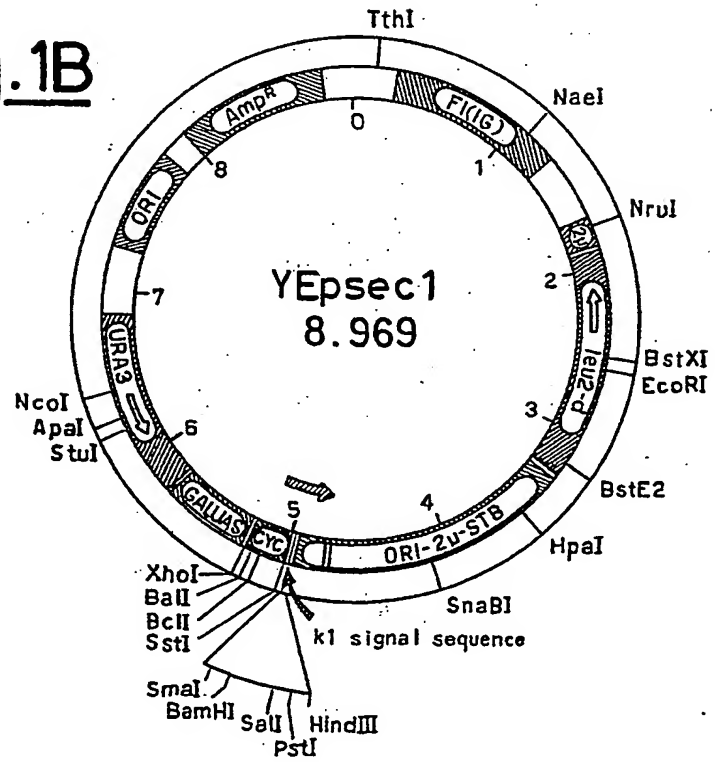



Fig. 1A

Fig. 1B




Met Asn Ile Phe Tyr Ile Phe Leu Phe Leu Leu Ser Phe Val

5'...GAGCTC ATG ATT ATA TTT TAC ATA TTT TTG TTT TTG CTG TCA TTC GTT

Glu Gly thr arg gly Ser Leu Asn Cys Thr

CAA GGT acc cgg gga TCA CTG AAC TGC ACG...3'

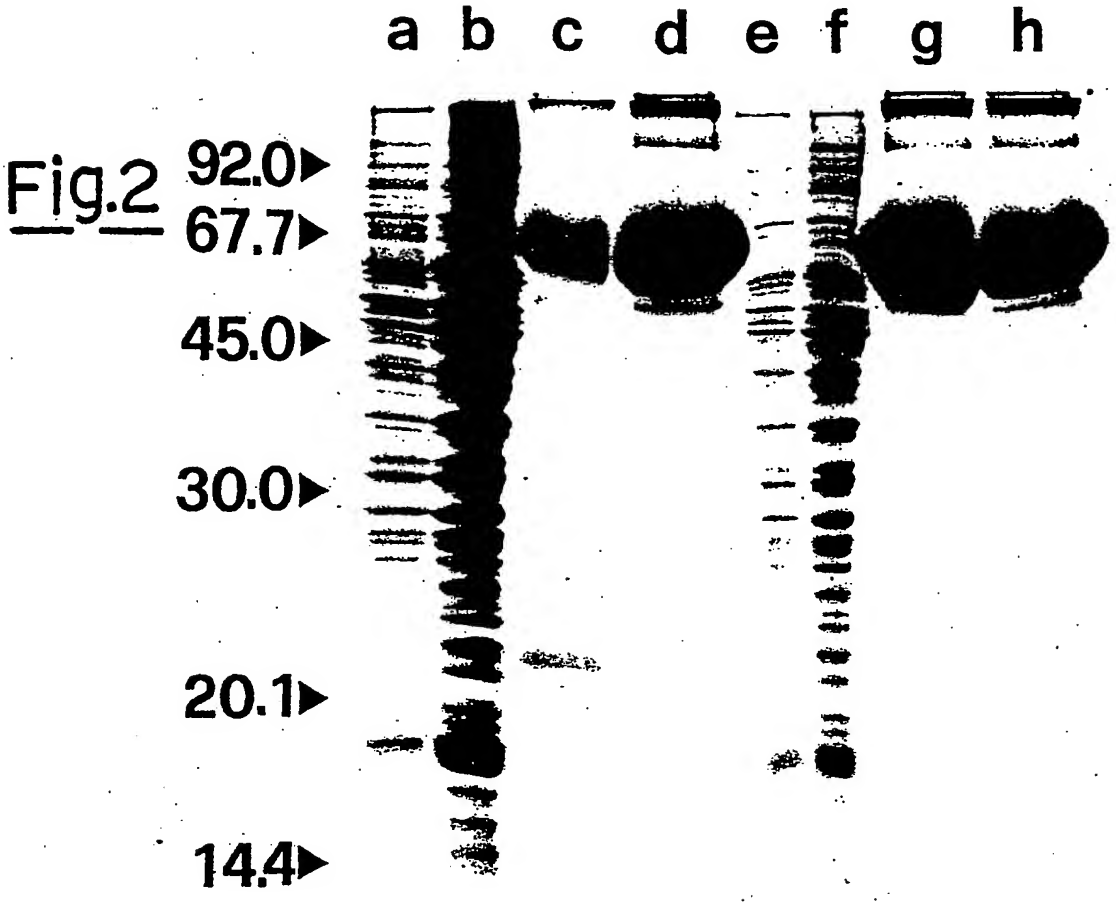



Fig.2

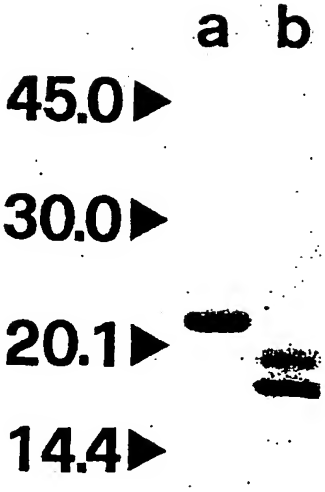
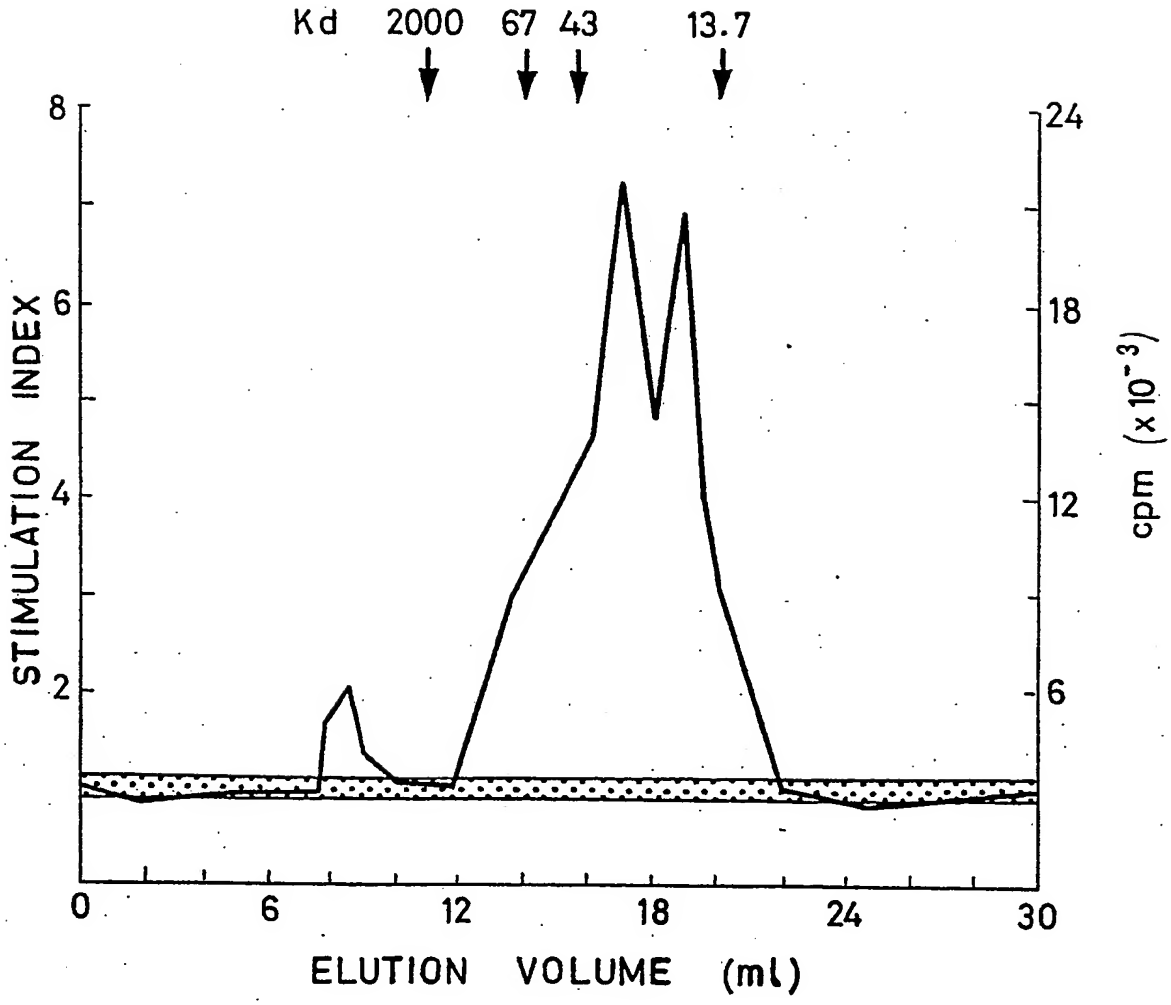



Fig.3

Fig.4


Kd
2000
67
43
13.7
8
6
4
2
STIMULATION INDEX
24
18
12
6
cpm (x10⁻³)
0
6
12
18
24
30
ELUTION VOLUME (ml)